Trop J Nat Prod Res, April 2018; 2(4):198-202 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

In vivo Evaluation of Antiplasmodial Properties of *Myosotis scorpioides L.* (*Boraginaceae*) Extract in Albino Mice Infected with *Plasmodium berghei*

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ARTICLE INFO

Article history:
Received 11 February 2018
Revised 03 April 2018
Accepted 07 April 2018
Published online 07 April 2018

ABSTRACT

Myosotis scorpioides L commonly called water forget-me-not or true forget-me-not has been claimed by Traditional Medicine Practitioners (TMP) in Adamawa State to cure malaria. It is a herbaceous perennial flowering plant in the borage family (Boraginaceae). The present study was undertaken to scientifically verify the claimed ethnomedicinal use of the plant as antimalarial. The crude powdered sample (whole plant) of *Myosotis scorpioides* was extracted with methanol in a Soxhlet extractor. The concentrated extract was screened for the presence of secondary metabolites, tested for the median lethal dose (LD_{50}) and antimalarial activity using Swiss albino mice. The result of the phytochemical screening indicated the presence of alkaloids, terpenes, tannins, flavonoids, saponins and anthraquinones. The result of the suppressive test (early malaria infection) showed a significant percentage suppression compared to the control with values of 49.91%, 56.72%, and 65.63% at the doses of 100 mg/kg, 150 mg/kg, and 250 mg/kg, respectively. The result of the prophylactic (residual malaria infection) tests showed a significant level of inhibition compared to the control (43.22%, 52.45%, and 85.70%) for the three doses. The curative (established malaria infection) tests also showed a significant level of parasite suppression compared to the control with percentage suppression of 66.73%, 70.20%, and 73.96% at 100, 150, and 250 mg/kg, respectively. The present study has validated the use of *Myosotis scorpioides* as remedy to malaria infection by the traditional medicine practitioners in Adamawa, State Nigeria.

***Keywords*:** Antimalarial, *Myosotis scorpioides*, *Plasmodium berghei,* Albino Mice.

Introduction

Malaria is a devastating disease that impacts huge health and economic burden on low-income countries in endemic regions.[1] Human malaria is caused by single-celled parasites, belonging to the genus Plasmodium.[2] In its different specific and clinical guises, malaria is one of the strongest selective forces to have shaped our recent evolution.[3] There are renewed attempts to control and eventually eradicate what remains one of the world's biggest killer disease.[4] According to a report by World Health Organization (WHO), Africa is home to 90% of malaria cases and 92% of malaria deaths, the region accounted for 76% of malaria cases and 75% deaths globally.[5] Malaria remains a major killer of children under five years old, taking the life of a child every two minutes. Thirteen countries (sub-Saharan Africa) – account for 76% of malaria cases and 75% deaths globally.[6] WHO in 2015 reported that nearly half of the world's population was at risk of Malaria and that there were 212 million cases of malaria in 2015 and 429000 deaths.[7] Malaria is common in tropical and subtropical regions because rainfall, warm temperatures, and stagnant waters provide an environment ideal for mosquito larvae survival.[8] Malaria is typically diagnosed by the microscopic examination of blood using blood films, or with antigen-based rapid diagnostic tests.[9] Modern techniques that use the polymerase chain reaction to detect the parasite's Deoxyribonucleic acid (DNA) have also been developed, but these are not widely used in malaria-endemic areas due to their cost and complexity.[10] An indispensable part of controlling malaria is the capability of treating the disease effectively, despite the ability of this highly mutable parasite to develop resistance to all classes of antimalarial. Resistance to antimalarial medicines is a threat to global efforts to control and eliminate malaria.[11] This resistance concerns numerous drugs but is thought to be most serious with chloroquine (CQ), the cheapest and most widely used drug to treat malaria.[12] The issue of resistance in malarial infection makes the development of novel drugs a necessity. An alternative source for discovering such drugs is natural products.[13] Improved access to effective malaria treatments has been a key contributing factor to the significant reduction in the malaria burden in recent years.[14] Search for the efficacy of plant derived antimalarial treatments is a top priority for malaria-endemic countries and the global malaria community.[15]

The requirements for new antimalarial drugs according to a review include; rapid efficacy, minimal toxicity and low cost.[16] Immediate prospects for drugs to replace chloroquine (CQ) and sulphadoxine pyrimethamine (SP) include amodiaquine (a CQ-like quinoline) and chlorproguanil dapsone (LapDap, another antifolate combination that inhibits the same enzymes as SP).[17] These replacements were thought to provide a few years of efficacy, particularly in Africa.[18] High on the list of mid-term replacements were artemisinin derivatives. However, these drugs have very short half-lives, which necessitated their use in combination with a longer-acting drug. Clearly, additional new drugs were urgently needed.[19] It became imperative to avoid an ever-increasing toll of malaria on tropical areas by putting into place a strategic action plan for the discovery and development of novel

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Citation: Zakari A, Kubmarawa D, Isyaka SM, Kendeson CA, Baba EB. *In vivo* Evaluation of Antiplasmodial Properties of *Myosotis scorpioides L.* (*Boraginaceae*) Extract in Albino Mice Infected with *Plasmodium bergei*. Trop J Nat Prod Res. 2018; 2(4):198-202. doi.org/10.26538/tjnpr/v2i4.8

antimalarial compounds that are not encumbered by pre-existing mechanisms of drug resistance.[20]

Infusions of the bark of Chincona plant (Rubiaceae were used to treat human malaria as early as 1632. The first antimalarial drug, quinine, was extracted from the bark of this tree. Since then herbal medicines have been used to treat malaria for thousands of years in various parts of the world.[21] There are economic benefits that will be gained from reduced malaria morbidity and mortality. Accordingly and in an effort to control malaria based on the recent report, about 510 million insecticide-treated nets (ITN) were distributed in sub-Sahara Africa.[22] The ethnomedical approach to the search for new anti-malarial drugs from plant sources has proved to be more predictive, where the most important modern anti-malarial drugs are derived from the medicinal plants known to have ethnomedical standing.[23] Treatment failure and/or resistance has been documented to all classes of antimalarial drugs, including the artemisinin derivatives.[24] This became dangerous for the patient and also for the community and it is a major threat to malaria treatment, control and eradication.[25] Antimalarials are used in three different ways: prophylaxis, treatment of falciparum malaria, and treatment of non-falciparum malaria. Prophylactic antimalarials are used almost exclusively by travelers from developed countries who are visiting malaria-endemic countries. The antimalarials in common use come from the following classes of compounds: the quinolines (chloroquine, quinine, mefloquine, amodiaquine, primaquine), the antifolates (pyrimethamine, proguanil and sulfadoxine), the artemisinin derivatives (artemisinin, artesunate, artemether, arteether) and hydroxynaphthaquinones (atovaquine).[26]

Traditional medicine practitioners in Adamawa State, Nigeria, have used the decoction of the whole plant of *Myosotis scorpioides L*. to treat malaria.[27] However its efficacy as an antimalarial remedy has not been proven scientifically. The present study attempts to provide scientific justification of the claim by TMP.

Materials and Methods

Plant Collection

The plant sample was collected by Clifford Emmanuel a research assistant from Girei, Adamawa State in March 2016. The plant was authenticated by a botanist in the Department of Biological Sciences Modibbo Adama University of Technology Yola.

Plant Preparation

The plant sample was washed thoroughly with distilled water, air – dried under shade, ground into fine powder and weighed.

Extraction

The plant sample (200 g) was extracted with hot methanol using 1 L Soxhlet extractor in batches of 50 g at a time.

Phytochemical Screening

Phytoconstituents which include alkaloids, tannins, steroids, saponins, flavonoids, phenols, anthraquinones and glycosides were screened using a modified method as previously reported by Mariod *et al*., 2011.[28]

Animals

Ninety 4-week-old albino mice (18 – 34 g) were obtained from the National Veterinary Research Institute (NVRI) Vom in Jos Plateau State, Nigeria.

Laboratory Animals Use, Care and Ethical Approval

The experimental procedures relating to the animals were authorized by the Ethical committee of National Institute for Pharmaceutical Research and Development (NIPRD), Abuja-Nigeria before starting the study and were conducted under the internationally accepted principles for laboratory animal use and care (EEC Directive of 1986; 86/609/EEC).[29]

Acute toxicity test (LD_{50})

The oral acute toxicity tests were carried out in mice using modified Lorke's method.[30]

Parasite inoculation

Mice infected with *P. berghei* was obtained from National Institute for Veterinary Research (NVRI) Vom in Jos - Nigeria. Standard inoculums of 1 x 10^7 *P. berghei* infected erythrocytes in 0.2 mL were prepared by diluting the infected blood with 0.9% normal saline. The mice were inoculated by intraperitoneal injection with a blood suspension (0.2 mL) containing 1 x 10^7 parasitized erythrocytes.[31] The parasite was maintained by serial passage of blood on a weekly basis from infected to non-infected mice.[32]

Suppressive Test

The Peter's 4 days suppressive test against chloroquine sensitive *Plasmodiurn berghe*i NK 65 infection in mice were employed according to modified method of Peter, 1967.

Adult Swiss albino mice were inoculated by intraperitoneal (IP) injection of standard inoculums of the *Plasmodium berghei* with 1 x 10^7 infected erythrocytes. The mice were randomly divided into five (5) groups of six (6) mice per group and treated for 4 consecutive days with 5 mL daily administration of 100, 200 and 400 mg extract kg^{-1} b.wt. orally. Two control groups were used: Positive control was treated daily with 5 mg chloroquine kg^{-1} b. wt while the negative control was given 5 mL kg^{-1} b.wt normal saline. On day 5 of the experiment, blood was collected from the tail of each mouse and smeared on to a microscope slide to make a film. The blood films were fixed with methanol, stained with 10% Giemsa at pH 7.2 for 10 min and parasitaemia determined microscopically. The percentage suppression of parasitaemia were calculated for each dose level by comparing the parasitaemia in negative control with those of treated mice.[33]

Average % suppression = (A-B)/A

A = Average percentage parasitaemia in negative control group, and
B = Average parasitaemia in test group.

Evaluation of Schizontocidal Activity of the Plants Extracts on Established Infection (Curative or Rane test)

Evaluation of the potential of the methanol extract was carried out according to the method described by Ryley and Peters, 1970. The mice were infected intraperitoneally with standard inoculums of l x10^7 *Plasmodium berghei* berghei NK 65 infected erythrocytes on the first day (day 0). Seventy-two hours (72 h) later, the mice were divided into 5 groups of six mice each. The groups were orally treated with 5 mL each of the plant extract at 100, 200 and 400 mg kg^{-1} day^{-1}. Chloroquine (5 mg kg^{-1} day^{-1}) was given to the positive control and an equal volume of distilled water was given to the negative control group. The treatment was carried out once daily for 5 days and blood smears were collected and examined microscopically to monitor the parasitaemia level.[34]

Evaluation of the Prophylactic Activity of the Plants Extracts (repository test)

Evaluations of the prophylactic potential of extracts of the plants were carried out according to the modified method of Peter 1967. Adult mice was randomized into 5 groups of six mice each. Group 1 were given 5 mL distilled water kg^{-1} b. wt. orally. Groups 2, 3, and 4 were given 100, 200, and 400 mg extract kg^{-1} b. wt, respectively. Group 5 were given 5 mg chloroquine (CQ) kg^{-1} b. wt intraperitoneally. Treatments were initiated on day 0 and day 4 then, the mice were all infected with the parasite. Blood smears were made from each mouse 72 h after treatment and increase or decrease in parasitaernia were determined as above.[35]

Statistical Analysis

The results were analyzed using Windows Excel 2013 softwares Inc. and presented as mean ± standard error of mean (SEM) and were subjected to one-way analysis of variance (ANOVA). Differences between means were considered significant at $P < 0.05$.[36]

The percentage parasitemia was calculated using the formula:

$$\% \text{ Parasitemia} = \frac{\text{Total number of parasitized erythrocytes}}{\text{Total number of erythrocytes counted}} \text{ X } 100$$

Average percentage of chemo suppression was calculated using the formula:

$$\% \text{ Suppression} = \frac{\text{Parasitemia in negative control} - \text{Parasitemia in test group}}{\text{Parasitemia in negative control}} \text{ X } 100.$$

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Results and Discussion

In vivo evaluation of antimalarial compounds typically begins with the use of rodent malaria parasites. Of these, *P. berghei*, *P. yoelii*, *P. chabaudi* and *P. vinckei* have been used extensively in drug discovery and early development. Rodent models have been validated through the identification of several antimalarials including mefloquine, halofantrine and artemisinin derivatives. In view of their proven use in the prediction of treatment outcomes for human infections, these models remain a standard part of the drug discovery and development process.[37] Individual species and strains have been well characterized, including duration of cycle, time of schizogony, synchronicity, drug sensitivity and course of infection in genetically defined mouse strains.[38] The most widely used initial test, which uses *P. berghei* or less frequently *P. chabaudi*, is a four-day suppressive test in which the efficacy of four daily doses of compounds is measured by comparison of blood parasitaemia (on day four after infection) and mouse survival time in treated and untreated mice.[39] Rodent infection is typically initiated by needle passage from an infected to a naïve rodent via the intraperitoneal or preferably the intravenous route, often using a small inoculum (typically in the range of 10^6-10^7 infected erythrocytes).[40]
The percentage yield of the methanol extract of *Myosotis scorpioides was* 22%. Preliminary phytochemical screening of the methanol extracts of *Myosotis scorpioides* revealed the presence of alkaloids, phenols, flavonoids, saponins and tannins as supported by recent literature.[41] The result of the antimalarial investigation of the plant extract showed a significant percentage suppression compared to the control with values of 49.91%, 56.72%, and 65.63% at the doses of 100 mg/kg, 150 mg/kg, and 250 mg/kg, respectively (Table 4). The result of the prophylactic (residual malaria infection) tests showed a significant level of inhibition compared to the control (43.22%, 52.45%, and 85.70%) for the three doses. The curative (established malaria infection) result showed a significant effect with values of 15.06 ± 6.65, 13.49 ± 5.64, and 11.79 ± 5.08 at doses of 100 mg/Kg, 150 mg/Kg and 250 mg/Kg, respectively compared to the value 45.27 ± 13.48 for the normal saline (negative control) (Table 5). The results of this investigation showed that the methanol extract of *Myosotis scorpioides* whole plant contain substances with antimalarial properties that showed suppressive, prophylactic and curative effects on *P. berghei*. The result of the study also indicated that the parasite clearance ability of the extracts was time-dependent (Figure 2). This was indicated by the observation that mice treated before the infection had lower parasite count and survived better than those treated after the infection.

Table 1: Phytochemical Screening of *Myosotis scorpioides*

Phytochemical Constituents	Inference
Indole alkaloid	+
Tropane alkaloids	+
Quinoline alkaloids	+
Morphane alkaloids	-
Steroids	+
Flavonoids,	+
Saponins,	+
Tannins	+
Phenols	+

+ indicates presence of phytoconstituent; - indicates absence of phytoconstituent.

Table 2: Acute toxicity result of *Myosotis scorpioides* Extracts.

Dose mg/Kg	Log10dose	%mortality
10	1	0
100	2	0
1000	3	0
1600	3.2	66.7
2900	3.5	66.7
5000	3.7	100

Table 3: Prophylactic Effect of Methanol Extract of *Myosotis scorpioides* and Chloroquine against *P. berghei* Infection in Swiss Albino Mice.

Treatment	% Parasitemia	% Chemo-suppression
10% saline (control) (5 mL/kg)	23.07 ± 1.62	0
Extract 100 $mgKg^{-1}$	13.10 ± 0.71	43.22
Extract 150 $mgKg^{-1}$	10.97 ± 0.87	52.45
Extract 250 $mgKg^{-1}$	3.30 ± 0.94	85.70
Chloroquine (CQ) (5 mg/kg)	1.17 ± 0.16	94.93

NS = not significant, *P < 0.01 (significant).

Table 4: Suppressive Effect of Methanol Extract of *Myosotis scorpioides* and Chloroquine against *P. bergei* Infection in Swiss Albino Mice.

Treatment	% Parasitemia	% Chemo-suppression
10% saline (control) (5 mL/kg)	46.72 ± 6.23	0
Extract 100 $mgKg^{-1}$	23.40 ± 6.77	49.91
Extract 150 $mgKg^{-1}$	20.22 ± 5.23	56.72
Extract 250 $mgKg^{-1}$	16.06 ± 4.08	65.63
Chloroquine (CQ) (5 mg/kg)	10.52 ± 6.00	97.48

NS = not significant, *P < 0.01(significant).

Table 5: Curative Effect of Methanol Extract of *Myosotis scorpioide* and Chloroquine against *P. bergei* Infection in Swiss Albino Mice.

Treatment	% Parasitemia	% Chemo-suppression
10% saline (control) (5 mL/kg)	45.27 ± 13.48	0.0^{ns}
Extract 100 mgKg-1	15.06 ± 6.65	66.73*
Extract 150 mgKg-1	13.49 ± 5.64	70.20*
Extract 250 mgKg-1	11.79 ± 5.08	73.96*
Chloroquine (CQ) (5 mg/kg)	1.37 ± 0.17	96.97*

NS = not significant, *P < 0.01 (significant).

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

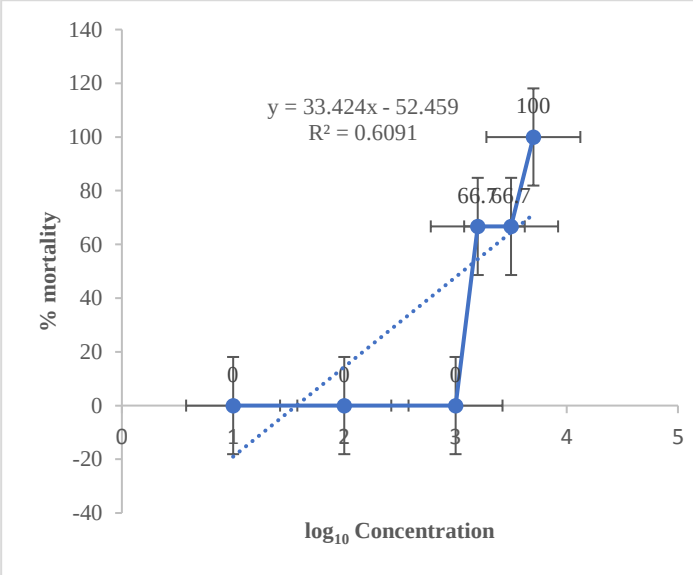

Figure 1: Graph of percentage mortality versus $\log_{10}$ Dose of *M. scorpiodes* Extract.

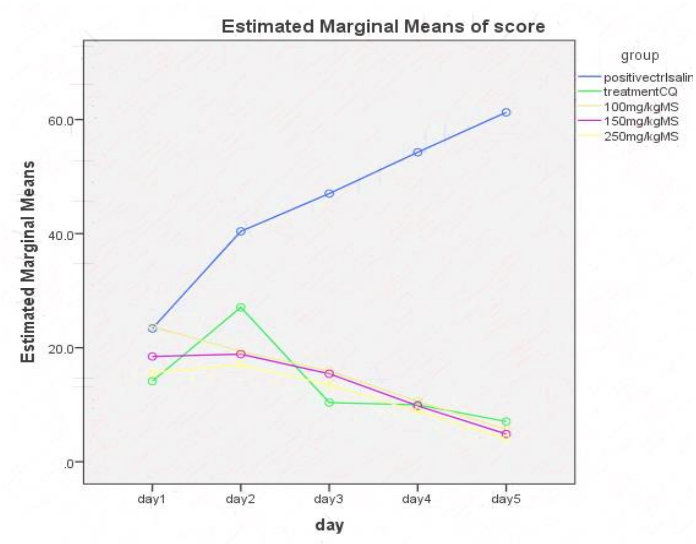

Figure 2: Curative Test Curve of *Myosotis scorpioides*.at 99% Confidence Level

Conclusion

The present study has partly justified the claim by traditional medicine practitioners in Adamawa State Nigeria on the antimalarial properties of *Myosotis scorpioides L*.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

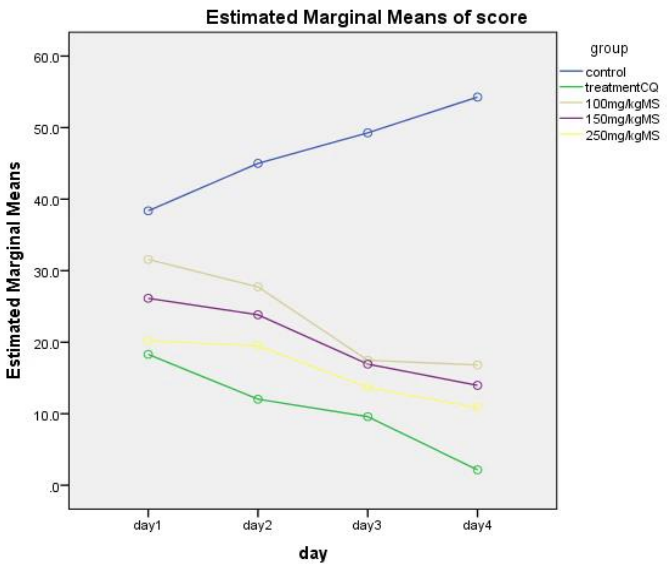

Figure 3: Suppressive Test Curve of *Myosotis scorpioides* at 99% Confidence Level.

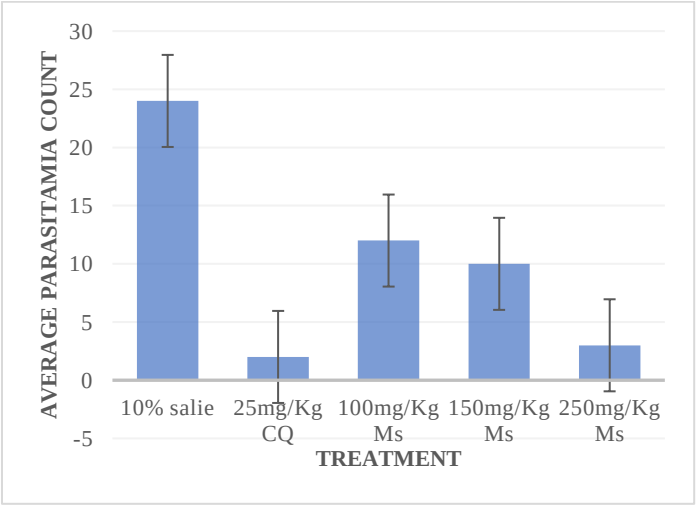

Figure 4: Column Chart of the Prophylactic Test of *Myosotis scorpioides* at 99% Confidence Level

Acknowledgements

Authors are grateful to TETFund – Nigeria for an intervention fund and to the Managements of Modibbo Adama University of Technology Yola and Federal University Kashere Gombe for their support.

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